ENGLISH TRANSLATION OF REFERENCE 1

REAGENT FOR ASSAY OF ANTIBODY AGAINST REDUCED ANTIGEN AND METHOD FOR DETERMINATION USING THEM

Field of invention

The present invention relates to assay for identifying the presence of an antibody having immunological reactivity to a hepatitis C virus (HCV) antigen in sample, in particular to converting cysteine residue existing in said antigen into a reduced form or reduced derivative or retaining it to detect the antibody having a specific reactivity to said reduced antigen or a derivative thereof. NS3 domain of HCV genome, in particular, 33C antigen in which a cysteine residue is converted into a reduced form or a reduced derivative or retained being its reduced form is useful as a reagent for detection of antibody in a body fluid of individual infected by a hepatitis C virus (HCV). In particular, the present invention relates to a reagent for determining to high sensitively and correctly determine an antibody against a hepatitis C virus (HCV).

Background of the invention

It has been found that acute viral hepatitis is caused by a virus such as hepatitis A (HAV) virus and hepatitis B virus (HBV). However acute viral hepatitis which an antibody against those HAV or HBV, cytomegalovirus, Epstein-Barr virus are undetectable has been clinically found, and it was proposed to call it non-A non-B hepatitis (NANBH). It has been successful to develop excellent tests for HBV, therefore nowadays the great majority of post-transfusion hepatitis have been non-A non-B hepatitis, from that point of view it has been desired to develop an effective test of non-A non-B hepatitis.

In such a situation, non-A non-B hepatitis factor cloned from chronic non-A non-B hepatitis infected chimpanzee by gene recombinant technology was called hepatitis C virus (HCV) (EP-A-0318216), EP-A-0388232).

As method for diagnosing the infection with hepatitis C virus (HCV), HCV antibody measurement system using C100-3 antigen was developed by Chiron Corporation, USA in 1988. It has been reported that said antigen called C100-3 contains 154 amino acid residues derived from human superoxide dismutase (SOD), 5 amino acid residues derived by expression of a synthesis DNA adapter containing EcoRI restriction enzyme site, 363 amino acid residues derived by expression of cDNA fragment from cloned HCV genome, and 5 amino acid residues being in 5 carboxy-terminal derived from MS2-cloning vector DNA in its amino-terminal.

In 1991, HCV antibody measurement system was developed, having the superior sensitivity and detection rate using core domain and C33 antigen coded by NS3 domain unduplicating C100-3 antigen being structural domain on HCV genome. Furthermore, the use of an antigen coded in NS4 domain has been tried. In order to determine those HCV antibody, methods such as aggregation method using erythrocyte or latex particle as an antigen supporting carrier, and immunometric method using beads, tube or plate as an antigen immobilizing carrier have been used.

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However, there was a problem that an activity of antigen was rapidly decreased during a process of immobilization of antigen on carrier or a preservation of reagent prepared, a antigen antibody reaction could not be sufficiently proceeded, thus a sensitivity of measurement did not sufficiently increase, furthermore a reproducibility of sensitivity was deteriorated for that an activity of antigen changed with time. The present inventors confirmed that the problem of a decrease of sensitivity in the HCV antibody measurement can be prevented by addition of a reducing agent, in particular thiol protecting agent to HCV measurement system, further that the treatment with a reducing agent did not affect adversely such measurement system, based on a discovery that the problem of a defective of sensitivity in the HCV antibody measurement system was caused by a natural oxidation of cysteine contained in a protein coded in NS3 domain on HCV antibody, in particular HCV genome, and by conversion of the cysteine to disulfide bond. Thus, the present inventors provided a composition for determining HCV antibody in a specimen by the immunological techniques, in which a reducing agent is incorporated into said HCV antibody or a peptide containing reagent having the substantially equivalent action thereto; or a composition in which a HCV antigen immobilized onto a carrier or a peptide having the substantially equivalent action thereto is treated with a reducing agent.

While further proceeding with the study using such reagents, it was found that an antibody against a reduced cysteine contained in protein coded in NS3 domain on HCV genome in HCV positive specimen of a body fluid from an individual infected by hepatitis C virus (HCV) indicated a far higher titer than that against an oxidized cysteine contained therein.

Therefore, it is useful in detecting HCV positive specimen to use a system stably determining an antibody against a protein part containing a reduced cysteine residue of a protein coded in NS3 domain on HCV genome (hereinafter, simply called as "anti-reduced HCV antibody"). Previously, a reducing agent has been incorporated into the peptide containing reagent, or a HCV antigen immobilized on a carrier has been treated at a certain time with a reducing agent. No system surely detecting only anti-reducing HCV antibody had been known. And, problem that the protein part containing the cysteine residue once reduced changed to an oxidized form could not be resolved.

Disclosure of invention

As a result of study based on idea that a measurement system which can stably detect at least said anti-reduced antibody would be extremely useful for detecting HCV positive specimen, the present inventors found that it was only necessary to enable a protein coded in NS3 domain on HCV genome, in particular a cysteine residue of 33C antigen to react with only said anti-reduced HCV antibody, and completed the present invention. Thus, the present invention relates to a composition for determining a HCV antibody by an immunological method, which comprises an antigen therefor or a peptide having substantially equivalent action thereto. In said composition, the cysteine residue in the protein antigen encoded by NS3 region on HCV genome (hereinafter, referred as "NS3 antigen") or peptide having substantially equivalent action thereto (hereinafter referred as "NS3 related peptide") is retained or converted so that it can react immunologically and specifically with an antibody reacting immunologically and specifically with a protein part of said NS3 antigen or NS3 related peptide in which a reduced cysteine residue is

contained (hereinafter referred as "anti-deduced HCV antibody"). Also the present invention relates to a method for preparing said composition.

Additionally, the abbreviation of NS3 related antigen includes NS3 antigen and NS3 antigen related peptide. It is found that said anti reduced HCV antibody is presence at far higher titer in HCV positive specimen than anti-oxidized HCV antibody, and sensitivity or reliability of measurement can be enhanced by using an antigen specifically reacting with said anti-reduced HCV antibody as a reagent. The anti-oxidized HCV antibody also refers to an antibody having reactivity against NS3 antigen in oxidation state herein.

Brief description of the drawings

Figure 1 shows the effects of an inert gas in a bottle. "He" refers to "in the presence of helium", and "Cont." refers to "control".

Figure 2 shows the relation between the sensitivity in each HCV antigen sensitized erythrocyte and HCV antibody positive serum. The vertical refers to Number.

Figure 3 shows the relation between the sensitivity in each HCV antigen sensitized erythrocyte and HCV antibody positive serum. The vertical refers to Number.

Figure 4 shows the correlation between the results in figure 2 and figure 3.

Figure 5 shows the correlation between the results in figure 2 and figure 3.

Detailed description of the invention

It is considered that a cysteine residue existing in NS3-related antigen can be converted into the residue having the immunological and specific reactivity to an anti-reduced HCV antibody or made to retain such residue by a treatment selected from the group consisting of following (1) to (6). In sum, all that is required is that such NS3-related antigen is converted into the antigen having the immunological and specific reactivity to such anti-reduced HCV antibody, or that it is the antigen retaining the immunological and specific reactivity to such anti-reduced HCV antibody:

(1) Treating NS3-related antigen with a reducing agent to convert a cysteine residue existing therein into its reduced form, and maintaining the reduced NS3-related antigen in a dried state, in an inert gas atmosphere or in the presence of a deoxygenating agent;

(2) Modifying a thiol group of a cysteine residue existing in NS3-related antigen with a protecting or modifying agent for thiol group such as iodoacetamide, iodoacetic acid, p-chloromercuribenzoic acid, methyl iodide or a metal such as mercury, iron, lead and the like;

(3) Modifying a cysteine residue existing in NS3-related antigen by genetic recombination techniques such as site-directed mutagenesis to prepare a variant recombinant NS3 antigen or a NS3 antigen-related peptide;

(4) Treating NS3-related antigen with a reducing agent to convert of a cysteine residue existing therein into its reduced form, and removing the reducing agent, and then maintaining the reduced NS3-related antigen in the presence of a

antioxidant until just prior to use;

(5) Cleaving a disulfide bond (-S-S-) existing in NS3-related antigen, and treating with an enzyme such as disulfide reductase and the like that can form the thiol group; and

(6) Treating NS3 antigen or NS3 antigen-related peptide with a reagent having an affinity for a cysteine residue existing in NS3-related antigen.

The antigen used in the present invention has a cysteine residue, in particular a reduced cysteine residue.

JP-A-02-500880, EP-A-0318216 and EP-A-0388232 disclose a clone having DNA sequence obtained by cloning a hepatitis C virus (HCV) genome, an antigen obtained using such disclosed sequence and having the cysteine residue is considered to be one of the antigen used in the present invention.

Above references describe C100-3 antigen expressed by C100-3 clone, it has been reported that such antigen called C100-3 contains 154 amino acid residues derived from human superoxide dismutase (SOD), 5 amino acid residues derived by expression of a synthesis DNA adapter containing EcoRI restriction enzyme site, 363 amino acid residues derived by expression of cDNA fragment from clonal HCV genome, and 5 amino acid residues being in 5 carboxy-terminal derived from MS2-cloning vector DNA in its amino-terminal. An antigen containing 1 to 150th amino acid residues of HCV sequence is known as HCV-core antigen. An antigen containing 1192 to 1457th amino acid residues of HCV sequence is a polypeptide from NS3 domain expressed by clone 33, which polypeptide is known as 33C antigen. An antigen containing 1676 to 1931th amino acid residues (comprising 256 amino acid residues) of HCV sequence is polypeptide from NS4 domain.

An antigen used in the present invention includes a polypeptide from NS3 domain, in particular 33C antigen or the antigen having the substantially equivalent action thereto. The activity of antigen refers to an ability to cause a specific antigen-antibody reaction, in particular to a activity of a specific antigen reacting with specific antibody in a specimen in the immunological techniques, and to an ability to react with an anti-reduced HCV antibody herein. A recombinant antigen being an expression product created by gene technological method or a synthesis peptide can be used in the present invention without particular limitation if having such nature. The antigen used in the present invention being the expression product created by gene technological method, i.e. the antigen produced by gene recombinant techniques is a recombinant antigen obtained by for example applying genetic recombination techniques using enzyme from DNA sequence obtained by cloning from a virus such as a natural hepatitis C virus or known genome sequence of HCV, or by expressing a chemical synthetic DNA sequence in microorganism, animal, plant, insect or cultured cell thereof. An example of such recombinant antigen may be at least one of the recombinant proteins (recombinant protein) expressing the different antigen domain of HCV genome. The antigen produced by genetic recombination techniques used in the present invention is preferably obtained as fused protein. In particular, in above treatment (3) of present invention the variant recombinant NS3 antigen or NS3 antigen-related peptide (variant recombinant NS3 related antigen) can be synthesized for example according to site-directed mutagenesis (R. Wu and L.

Grossman, Methods in Enzymology, Vol. 154, Academic Press, New, 1987, section IV, p.3291 or later, for example p.329, 350, 367, 382). Said cysteine residue can undergo the treatment such as excision such as deletion or substitution with other amino acid, furthermore excision of the adjacent sequence or substitution with other sequence. Those determinations can be carried out by an experiment. Such a cysteine residue may be replaced with a neutral amino acid such as glycine, valine, alanine, leucine, isoleucine, tyrosine, phenylalanine, histidine, tryptophan, serine, threonine, methionine, and the like.

In more preferably, such recombinant antigen may be obtained as a fused protein of CKS, SOD, β-galactosidase, glutathione, S-transferase, IgG-binding domain of protein A, maltose binding protein or related proteins thereof. The example includes a gene product of CKS fused protein expression system using E. coli as a host cell, especially a fused protein with a protein having initial 239 amino acid sequence of total 248 amino acid sequence of CKS, and a gene product of SOD fused protein expression system using a yeast as a host cell. The recombinant antigen obtained by CKS fused protein expression system is described in for example JP-A-04-253998 and JP-A-04-281792, furthermore includes for example pHCV-23 recombinant antigen, pHCV-29 recombinant antigen, pHCV-31 recombinant antigen, pHCV-34 recombinant antigen, pHCV-45 recombinant antigen, pHCV-48 recombinant antigen, pHCV-49 recombinant antigen, pHCV-50 recombinant antigen, pHCV-51 recombinant antigen, pHCV-57 recombinant antigen, pHCV-58 recombinant antigen, pHCV-101 recombinant antigen, pHCV-102 recombinant antigen, pHCV-103 recombinant antigen, pHCV-104 recombinant antigen, pHCV-105 recombinant antigen, pHCV-107 recombinant antigen, or a peptide obtained by treating them with peptidases or chemical cleaving agents and processing, for example a peptide which the CKS gene-derived peptide part is cleaved. The fused sites may be cleaved by chemical treatment such as cyanogen bromide, hydroxylamine, formic acid, acetic acid-pyridine solution or 2-(2-nitrophenylsulfenyl)-3-bromoindole-skatole, or with enzyme such as trypsin, lysylendoprotease, Xa factor, thrombin, human plasma kallikrein.

The transformed cells thus obtained such as E. coli and yeast can be broken by mechanical means such as homogenizer with glass beads or alumina beads, waring blender, french press or ultrasonic breaker, or lysed by enzymatic means using enzyme such as lysozyme, physical means such as freezing and thawing method or osmotic pressure, or chemical means using surfactant such as sodium dodecyl sulfate (SDS), Tween or Triton X, organic solvent such as acetone or butanol or chelating agent such as EDTA. When the cell is broken or lysed, a protease inhibitor may be added in suspension. An ultrasonic treatment can be used to break cells by use of an ultrasonic transducer generating 10 to 60kHz of sound waves (ultrasonic waves) to a suspension. Examples of such equipment include a type soaking ultrasonic transducer bar in a sample, a type treating by filling a cupped container fixed with ultrasonic transducer with sample, a cyclical type capable of consecutive processing, furthermore a type treating a sample with beads, and the like. Said ultrasonic apparatuses are commercially available from Otake Seisakusyo corporation, central scientific commerce, inc., and Seiko Instruments Inc.. The ultrasonic treatment is carried out for time sufficient to fragmentize a cell, such time can be selected as appropriate depending on a amount of a used sample and a used output, usually a range from about 1 minute to about 2 hours. The transformed cell lysate containing antigen thus obtained can be further isolated

and purified by for example salting-out using protein precipitating agent such as ammonium sulfate, precipitation using organic solvent such as ethanol, extraction using surfactant, dialysis, centrifugation such as density-gradient centrifugation, ultrafiltration, adsorption with ion-exchange resin, ion-exchange cellulose, ion-exchange sephadex, alumina or hydroxyapatite, column chromatography, electrophoresis, or affinity chromatography using dextran gel, polyacrylamide gel, polyethyleneglycoldimathacrylic acid gel, agarose gel, porous silica glass, molecular sieve, affinity chromatography using a monoclonal antibody, and the like, and can be processed appropriately for use. Those treatments may be combination of the appropriate methods. In those treatments, it can be carried out in a reduction state. For example, said treatments can be carried out using medium containing reducing agent or under the inert gas atmosphere to eliminate the contacts with oxygen or under the coexistence with deoxidant or in the presence of deoxidant. An example of reducing agents which may be used for reducing treatment is at least one selected from the group consisting of dithiothreitol, dithioerythritol, thioglycolic acid, cysteine, glutathione, 2-mercaptoethanol, 2-mercaptoethylamine, and a mixture thereof. In such treatment, particularly dithiothreitol, glutathionem and 2-mercaptoethanol are preferable.

An antigen used in the present invention may be a recombinant antigen being an expresstion product created by gene technological method or a synthesis peptide without particular limitation if having an ability of a specific reaction immunologically with above anti-reduced HCV antibody. In the present invention, if the antigen has thiol group of cystein or the disulfide-bond derived from it in its molecular, but it cannot affect an activity of said antigen, it is not intended as subject antigen for the treatment of the present invention.

The measuring object sample specimen used in the present invention includes, but are not limited to, biologic material such as whole blood, serum, plasma.

In the present invention, the reagent used for immunological measurement of a specific antibody in a specimen is prepared using the sensitized carrier obtained by immobilizing NS3-related antigen which has been subjected to a treatment selected from the group consisting of above (1) - (6) to a carrier such as insoluble carrier. According to the above method (1), in order to obtain the reagent of the present invention, for example, the reduced antigen can be immobilized to a carrier and then lyophilized, or the sensitized carrier obtained can be dispersed in an appropriate buffer under an inert gas atmosphere substantially without any reducing agent. The reagent obtained is stored under an inert gas atmosphere just prior to use. The reagent obtained can be used for measurement without any reducing agent during measurement. Said inert gas includes nitrogen gas, argon gas, helium gas, carbon dioxide, a mixed gas thereof and the like. If necessary, the antigen may be treated with at least one agent selected from the group consisting of, for example, dithiothreitol, dithioerythritol, thioglycolic acid, cysteinee, glutathione, 2-merucaptoethanol, 2-merucaptoethylamine and a mixture thereof prior to immobilizing the reduced antigen onto the carrier. Dithiothreitol, glutathione, 2-merucaptoethanol and the like are preferred for such treatment. In the present invention, above all or any treatment of sensitizing-treatment or immobilization, lyophilizing-treatment, preservation and measurement can be carried out under coexistence with a deoxidant or in the presence of a deoxidant. Said deoxidant includes inorganic iron powder, bisulfite salt, sulfite salt, thiosulfate salt and

pyrosulfite salt of alkali metal or alkaline-earth metal; organic ascorbic acid, hydroquinone, catechol and the like. Said deoxidant includes commercially available "ageless (brand name)". As used herein, the deoxidant differs from the reducing agent and the thiol protecting group above mentioned. As used hercin, the insoluble carrier includes a well known insoluble carrier, for example synthesized resin, a carrier made of a natural polymer such as nitrocellulose or a synthesized polymer, as well as a latex particle or an erythrocyte and the like.

According to the above method (2), for example, the antigen is treated with a thiol group modifying agent, and then the modified antigen is immobilized, followed by dispersed in an appropriate buffer to give the reagent of the present invention. Said thiol group modifying agent includes iodoacetic acid, iodoacetamide, halogenoacetic acid such as bromoacetic acid or amid thereof, 2-bromopropionic acid, N-ethylmaleimide, p-chloromercuribenzoic acid, methyl iodide, or metal such as mercury, iron, lead and the like, potassium tetrathionate, sodium tetrathionate, methyl methanesulfonate, 2-hydroxycthyldisulfide, S-acetylmercaptosucccinic acid anhydride, N, N'-o-phenylenedimaleimide, gultathione or N-oxide thereof and the like. According to the above method (4), for example the antigen is immobilized to a carrier and then the sensitized carrier is dispersed in an appropriate buffer in the presence of deoxidant (excluding reducing agent or thiol protecting group) to give the reagent of the present invention. In the present invention, the reagent used for immunological measurement of the specific antibody in an specimen by enzyme immunoassay (EIA), radioimmunoassay (RIA) or fluoroimmunoassay (FIA) is also prepared using the immobilized antigen obtained by immobilizing NS3-related antigen subjected to a treatment selected from the group consisting of above (1) - (6) to an insoluble carrier.

After HCV antigen is immobilized to insoluble carrier, the resulting antigen-coupled solid-phase can be subjected to the treatment selected from the group consisting of above (1) - (6), followed by immersed in the buffer to give the reagent of the present invention. In the present invention, the measurement of the antibody having the immunological reactivity to the antigen in a sample can be carried out by, if necessary, immobilizing the antibody participating in the antigen-antibody reaction to a carrier, and the sensitized carrier thus obtained can be contacted with a sample containing the antibody as the object of analysis. The antigen immobilized to the carrier is specifically coupled with the antibody in the analysis sample, and can be detected. As used herein, the insoluble or solid carrier includes a well known immobilized carrier, for example a carrier selected form polyethylene, polypropylene, polystyrene, styrene-butadiene copolymer, polyvinylchloride, polyvinyl acetate, polyvinyl alcohol, teflon; polyester, polyamide and polyurethane such as polyacetal, polyacrylamide, polymethacrylate, polyacrylate, styrene-methacrylate copolymer, polyglycidyl methacrylate, acrolein-ethylene glycol dimethacrylate copolymer; synthetic resin such as polyepoxy resin; cellulose ester or mixed cellulose ester of agar, agarose, cross-linking agarose, cross-linking alginic acid, cross-linking gua gum, paper, cellulose, cellulose nitrate, carboxy cellulose; polymer amino acid such as dextran, gelatin, chitin, collagen, cotton; nature or synthetic modified or unmodified polymer carbohydrate such as polysaccharide; polymer such as polymer carbohydrate, cross-linking derivative thereof; glass, for example activated glass, silica gel, alumina, kaolin, talc, silica-alumina, ceramic, carbon, barium sulfate, magnesium sulfate, and is in the form of particle, fine particle, membrane, bead, tube, plate, microplate, microtiter well, microtube, strip.

Additionally, an erythrocyte, a latex particle, an emulsion are included in the insoluble or solid carrier.

In the present invention, the measurement of the antibody having the immunological reactivity to the antigen in the sample can be carried out by radioimmunoassay, enzyme immunoassay, fluorescence immunoassay, chemiluminescence or bioluminescence immunoassay and agglutination immunoassay. Also, chemiluminescence immunoassay can measure by automation, and is a preferred method. In radioimmunoassay, the antigen leveled with enzyme immunoassay, chemiluminescence or bioluminescence immunoassay, or fluorescence immunoassay and the like, the radioactive material such as ^{125}I, ^{3}H, enzyme such as horseradish peroxidase, β-D-galactosidase, alkaline phosphatase; fluorescent pigment such as fluorescein; chemiluminescence pigment such as acrydinium esters; gold colloid, selenium colloid; or colored material such as colored latex particle, light-emitting material or coloring material can be used as a reagent. The reagent is specifically coupled with the antibody and/or the complex in the analysis sample, and then the radioactivity, enzyme activity, chemiluminescence or color thereof is measured to determine whether the antibody is present or absent in the sample. In the present invention, for example 4-hydroxyphenylacetic acid; 1,2-phenyleneziamine, tetramethylbenzidine; and enzyme reagent such as horseradish peroxidase, umbellipheryl galactosidase, nitrophenyl galactoside and β-D-galactosidase, umbellipheryl phosphate, nitrophenyl phosphate, NADP and alkaline phosphatase, glucose-6-phosphate dehydrogenase; radioactive material; fluorescence reagent, emission reagent and chemiluminescence reagent using fluorescein isothiocyanate, tetramethyl rhodamine isothiocyanate, acrydinium esters, emission lanthanide; colloid labeled reagent such as gold colloid, silver colloid, selenium colloid; magnetic reagent; hapten labeled antihapten-antibody detection reagent such as biotin labeled antibiotin-antibody can be used as a reagent for ditection.

In the present invention, chemiluminescent substances such as acridinium esters or fluorescent substances such as light-emitting lantanides are preferably used as labeling agents. Using such agents, automatic analysis can be carried out. In particular, chemiluminescence immunoassay using a reagent labeled with an acrydinium ester is preferred, and can be used in automatic analysis. Said acrydinium esters include N-alkyl or aryl acryziniun-9-caruboxylic acid ester described in JP-62-39598, JP-62-61969, JP-63-57572, JP-63-101368, JP-63-112564, JP-01-261461, GB1,461,877, US3,539,574. In particular, 10-alkyl, N-alkyl or aryl-sulfonyl-N-alkyl or arylsulfonylacrydinium-9-carboxamide, N-methylacrydinium-9-carboxylic acid ester described in JP-63-112564, US3,539,574 are included in the representative chemiluminescent labels. For acrydinium labels, the measurement can be carried out using a lumionometer by using a reagent to initiate emission, for example by treatment with hydrogen peroxide and a sodium hydroxide solution. For example, about 0.01% to about 0.1% of hydrogen peroxide solution and for example about 0.05N to about 0.5N of sodium hydroxide solution are used. Said light-emitting lanthanides include for example the chelatable light-emitting lanthanide having aminocarboxylic acid group described in EP0068875, US4,374,120, US4,283,382, US4,259,313, US4,352,751, US4,432,907, EP0103558. The measurement may be made easier by an exciting treatment with laser prior to measurement.

Of course, the labeling agents are not limited to above, and can be

appropriately selected from those known in the art depending on the purpose in consideration for the equipment used for measurement and the place where the measurement is carried out.

In the measurement method using an agglutination reaction, an agglutination reaction between a particulate antigen and a specific antibody is used. The agglutination reaction produces an observable agglutinate. In such a measurement method, an antigen is immobilized onto a particulate carrier such as an erythrocyte, a polystyrene particle, a latex particle, etc. For example, in order to detect an antibody in a sample, the particulate antigen is mixed and reacted with a sample, and then determine the existence of the antibody of interest, for example by observing an agglutination reaction in the aqueous medium. In the measurement using such an agglutination, a known concentration or a known amount of the antibody in the specific dilutions are added to the specific amount of the antigen, and then the degrees of the agglutination in the solutions are expressed in the reciprocal numbers of the dilution factors, and estimation can be carried out. Contrary, a known concentration or a known amount of the antigen in the specific dilutions are added to the specific amount of the antibody, and the degrees of the agglutination of the resulting solutions are expressed in the reciprocal numbers of the dilution factors, and estimation can be carried out. Furthermore the agglutination may be indirectly observed using an antibody to the antibody, i.e. a secondary antibody. The passive agglutination immunoassay is successfully used in for example the measurement of the antibody to HCV according to the present invention. Solid carriers, particulate carriers or labels can be coupled with an antigen by methods well known in the art, for example the physical adsorption or chemical bonding such as ion interaction, hydrophobic interaction, covalent bond and the like. The chemical bonding agents can be selected from those known in the art, and include, but are not limited to glutaric aldehyde, 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide, N, N'-o-phenylendimaleimide, N-succinimidyl 3-(2-pyridylthio)propionate, N-succinimidyl S-acetylmercaptoacetate, N-succinimidyl 4-(N-maleimidemethyl)cyclohexane-1-carboxylate, N-succinimidyl 6-maleimidehexanoate, N-succinimidyl 4-iodoacetylaminobenzoate, N-succinimidyl 3-(p-hydroxyphenyl)propionate, N-succinimidyl m-maleimidebenzoate, N-succinimidyl 4-maleimidebutyrate, N-succinimidyl (p-maleimidephenyl)acetate, N-succinimidyl 4-(p-maleimidephenyl)butyrate, and an activated ester of 6-maleimidecaproic acid, 2-bromoacetic acid, 2-iodoacetic acid, succinic acid, an activated ester of triazine, and a sulfonic acid ester derivative.

In the measurement of the present invention, a surfactant, a buffer, a dilute solution or a diluent, a blocking agent, a chelating agent, a preservative and the like may be used. The surfactant may be selected from anionic surfactants, cationic surfactants, amphoteric surfactants, and nonionic surfactants. The anionic surfactant includes an alkaline metal salt or alkaline earth metal salt of a higher fatty acid containing 12 to 18 carbon atoms, organic basic salt such as triethanolamine of a higher fatty acid containing 12 to 18 carbon atoms, and a sulfuric ester, alkylsulfonic salt and alkylarylsulfonic salt of higher fatty acid or higher alcohol containing 12 to 18 carbon atoms. The cationic surfactant includes a quaternary ammonium compound having alkyl group, aryl group, heterocyclic group. The alkaline metal includes sodium, potassium, lithium, and the alkaline earth metal includes calcium, magnesium and the like.

The amphoteric surfactant includes, but are not limited to polyamino-monocarboxylic acid, higher alkyl amino acid having 12 to 18 carbons, N-trialkyl substituted amino acid such as lauryl dimethylbetaine. The nonionic surfactant includes polyhydric alcohol ester of higher fatty acid containing 12 to 18 carbon atoms such as glyceryl monostearate, polyoxyethylene ester of higher fatty acid, sorbitan ester of higher fatty acid, ester of higher fatty acid with polyoxyethylene and sorbitan ether, ether of higher alcohol such as polyoxyethylene lauryl alcohol with polyoxyethylene, ether of polyoxyethylene with polyoxypropylene. A buffer, dilute solution or diluent includes water, phosphate buffer, tris(hydroxymethyl)aminomethane buffer, for example sodium chloride solution such as saline, N-(2-hydroxyethyl)piperazine-N'-(2-ethanesulfonic acid (HEPES) solution, piperazin-N,N'-bis(2-ethanesulfonic acid) (PIBES) solution, 3-(cyanohexylamino)-1-propanesulfonic acid (CAPS) solution, 3-(morpholino)propanesulfonic acid (MOPS) solution, N, N'-bis(2-hydroxyethyl)-2-aminoethanesulfonic acid (BES) solution, N-tris(hydroxymethyl)methyl-2-aminoethanesulfonic acid (TES) solution, N-(2-acetamide)-2-aminoethanesurufonic acid (ACES) solution, amino acid solution. Those may be used alone or as combination of two or more. The chelating agent includes ethylenediaminetetraacetic acid (EDTA), ethyleneglycol-bis(β-aminoethylether)-N, N, N', N'-tetraacetic acid (EGTA). Those chelating agent can be used in the range from about 0.01 mM to about 20 mM. The preservative includes for example sodium azide, ethylparaben. Additionally, to the measurement system of the present invention is added other materials selected from the group consisting a serum of various animals such as bovine serum, bovine serum albumin (BSA), fetal calf serum (FCS), goat serum, egg albumin, gelatin, various milk proteins such as skim milk, casein, casein resolvent and whey protein, polyvinyl alcohol, and polyvinylpyrrolidone. Those can be added in the range of about 0.01 % v/v to about 50 % v/v.

In the present invention, the lymphocyte fragment, for example human T-lymphocyte extract, E. coli extract, yeast extract, mouse cell medium extract may be also added. Those can be added in the range of about 0.001 % v/v to about 20 % v/v.

EXAMPLES

The following examples further illustrate, but do not in any way limit, the invention.

Example 1

A human erythrocyte for immobilization was washed with phosphate buffer (pH 7.4) three times before suspended in pH 5.7 of acetate buffer to 1 % v/v, a purified HCV 33C produced by gene recombinant techniques was added to 6μg/ml of final concentration, stirred for 1 hour at room temperature, then washed with phosphate buffer (pH 7.4) three times, lyophilized in phosphate buffer (pH 7.4) containing 7.5% of sucrose and 30ml of glutathione (GSH) to prepare 33C antigen sensitized erythrocyte.

The resulting 33C antigen sensitized erythrocyte was poured into the bottle comprising tris-hydrochloric buffer (pH 7.8) to obtain 1 % v/v of suspension. Said bottle comprising 33C antigen sensitized erythrocyte was stored with about two times and twenty times amount of deoxidant, ageless (brand name, Mitsubishi Gas Chemical Company, inc.) of oxygen concentration

in the closed space. The bottle comprising 33C antigen sensitized erythrocyte as control was left to stand in the absence of deoxidant in the opened space. The resulting 33C antigen sensitized erythrocyte suspension was left to stand for 0 to 9 days at 25 °C, and then compared sensitivity. In the comparison of sensitivity 33C antibody positive human serum was pre-diluted with said antibody-negative serum, used as the serum for management of the sensitivity. 25µl of phosphate buffer (pH 7.4) and 25µl of each concentration of serum for sensitivity calibration were dispensed to each well of microtiter plate, stirred for 30 seconds by mixer, left to stand for 1 hour at room temperature, and then the results were determined by visual inspection.

The results are shown in Table 1.

Table 1 efects of deoxidant on sensitivity

	Start	Day 2			Day 5			Day 7		
Amount of deoxidant		0	×2	×≥20	0	×2	×≥20	0	×2	×≥20
panel specimens	A	3	3	3	−	3	3	−	3	3
	B	1	3	3	−	3	3	−	3	3
	C	3	3	3	3	3	3	3	3	3
negative control		−	−	−	−	−	−	−	−	−
positive control		2+	2+	2+	2+	2+	2+	2+	2+	2+
reagent control		−	−	−	−	−	−	−	−	−

When control 33C antigen sensitized erythrocyte suspension was used, it was observed that the sensitivity was disappeared in A panel and B panel serum specimen on the 5th day. However, it was not observed that the sensitivity was changed in any panel serum of the group which the deoxidant was added.

Example 2

Helium gas was charged to a bottle for storage of 33C antigen sensitized erythrocyte, further tris-hydrochloric buffer (pH 7.8) was added. The lyophilized 33C antigen sensitized erythrocyte obtained in a manner similar to example 1 was used, said HCV antigen sensitized erythrocyte was added to said bottle to 1 % v/v through a syringe, 33C antigen sensitized erythrocyte was suspended. 33C antigen sensitized erythrocyte suspended in tris-hydrochloric buffer in a bottle without any inert gas such as helium gas was used as control. The resulting 33C antigen sensitized erythrocyte suspension was left to stand for 0 to 9 days at 25 °C, and then compared sensitivity in a manner similar to example 1.

The results are shown in figure 1. 33C antigen sensitized erythrocyte suspension in the helium gas-charged bottle sufficiently retained the sensitivity after 16 days, however 33C antigen sensitized erythrocyte suspension stored in air disappeared on the 5th day, substantially interfere with use thereof on the second day.

Example 3

The lyophilized 33C antigen sensitized erythrocyte obtained in a manner similar to example 1 was suspended to 4 % v/v in a bottle containing tris-hydrochloric buffer (pH 7.8), and then lyophilized.

Resulting 33C antigen sensitized erythrocyte was (1) resuspended in 110 mM of tris-hydrochloric buffer (pH 7.8) containing 1% of bovine serum albumin (BSA) and 6% of fetal calf serum (FCS), and then left to stand for 1 hour at room temperature, (2) resuspended in a manner similar to (1), and then left to stand for 4 days under the air atmosphere at room temperature, and (3) left to stand for 4 days under the air atmosphere at room temperature as (2), and then treated with 110 mM of tris-hydrochloric buffer (pH 7.8) containing 30 mM of glutathione, left to stand for 1 hour, and tested respectively. The serum indicating 5'NC-region-RT-PCR HCV RNA positive the serum indicating negative was used as specimen. The serum was prediluted with a serum being HCV antibody negative according to example 1, used as the serum for management of the sensitivity to measure.

The results are shown in Table 2. In both PCR positive and negative serum specimen, (1) a titer of antibody against a reduced antigen which may be measured with hemocyte for 1 hour after resusupension was higher the titer of antibody against a oxidized antigen which may be measured with hemocyte left to stand for 4 days after resuspension. Furthermore, it was observed that the reactivity of antigen similar to (1) was reappeared when the oxidized antigen was reduction-treated with glutathione solution.

Table 2 The titer of antibody against HCV 33C antigen

specimen number	PCR negative specimens			specimen number	PCR negative specimens		
	reduced 33C antigen (1)	oxidized 33C antigen (2)	reroduced 33C antigen (3)		reduced 33C antigen (1)	oxidized 33C antigen (2)	roreduced 33C antigen (3)
49	6	<3	6	46	6	<3	6
40	7	3	7	58	13	9	13
64	7	3	7	48	13	<3	13
59	7	4	7	7	15	9	14
51	7	<3	7	62	15	10	15
39	8	5	7	57	16	10	16
29	8	<8	8	9	>14	7	>14
26	9	7	9	13	>16	7	>16
15	11	5	11	10	>16	10	>16
34	11	5	10	11	>16	10	>16
55	11	5	11	84	>16	10	>16
58	11	<3	11	6	>16	11	>16
50	12	7	11	17	>16	11	>16
52	13	7	13	12	>16	12	>16
20	15	8	15	13	>16	12	>16
38	>14	8	>14	5	>16	13	>16
28	>14	9	>14	14	>16	14	>16
41	>14	10	>14	15	>16	14	>16
25	>16	14	>16	38	>16	14	>16
14	>16	15	>16	8	>16	15	>16

Example 4

A core antigen, C100 antigen and 33C antigen of HCV antigen were sensitized in a manner similar to example 1, prepared each HCV antigen sensitized erythrocyte. In a similar way, HCV PHA antigen sensitized erythrocyte was prepared using a mixture of core antigen, C100 antigen and 33C antigen as HCV antigen. It was observed that a cysteine residue in HCV 33C antigen sensitized erythrocyte was sufficiently a reduced type. The comparison of sensitivity was carried out using each HCV antigen sensitized erythrocyte according to example 1.

The results are shown in figure 2 and figure 3. Figure 2 (a) shows the results in HCV PHA antigen sensitized erythrocyte, Figure 2 (b) shows the results in HCV 33 antigen sensitized erythrocyte, Figure 3 (c) shows the results in HCV core antigen sensitized erythrocyte, and Figure 3 (d) shows the results in HCV C100 antigen sensitized erythrocyte. In the results in HCV PHA antigen sensitized erythrocyte and HCV 33C antigen sensitized erythrocyte, its distributions were well corresponded, therefore it was demonstrated that a reduced-33C antigen was significantly useful as reagent for determining HCV antibody.

Example 5

33C antigen sensitized erythrocyte obtained in a manner similar to example 1 was lyophilized in a phosphate buffer (pH 7.4) containing 7.5 % of sucrose and 10 mM of dithiothreitol (DDT) to prepare 33C antigen sensitized erythrocyte. The comparison of sensitivity was carried out using resulting 33C antigen sensitized erythrocyte according to example 1. The results were similar to those of example 1.

Example 6

A quarter inch of polystyrene beads were added to a phosphate buffer (pH 7.4) containing 7.5 % of sucrose, the purified HCV 33C antigen produced by gene recombinant techniques used in example 1 was added to 10 μg/ml of final concentration, left to stand for 1 hour at 37 °C. Then it was washed three times with a phosphate buffer (pH 7.4), then soaked in a phosphate buffer (pH 7.4) containing 7.5 % of sucrose and 20 mM of 2-mercaptoethanol (2-ME), followed by dried to give 33C antigen immobilized beads. 33C antigen immobilized beads obtained by soaking in a phosphate buffer (pH 7.4) with 7.5 % of sucrose and without 2-ME then drying were used as a control immobilized beads.

One of stored 33C antigen immobilized beads was added to a reaction tube charged with 200 μl of tris-hydrochloric buffer (pH 7.8), 10 μl of each concentration of serum for sensitivity calibration was added, reacted for 1 hour at 37 °C. After the reaction was completed, the reaction was washed three times with brain, 200 μl of peroxidase-labeled human immunoglobulin G antibody was added, reacted for 1 hour at 37 °C. After the reaction was completed, the reaction was washed three times with brain, 1ml of substrate solution containing orthoethylenediamine and hydrogen peroxide were added, reacted for 30 minutes at room temperature, then 1 ml of 1N sulfuric acid solution was added, after the reaction was stopped an absorbance was determined at 490 nm. 2-ME treated beads were significantly sensitive.

Example 7

33C antigen immobilized beads were prepared by treating in a manner

similar to example 6, except for soaking to a phosphate buffer (pH 7.4) containing 7.5 % of sucrose and 40 mM of glutathione (GSH) then drying. The comparison of sensitivity was carried out using resulting 33C antigen immobilized beads in a manner similar to example 6. The results were similar to those of example 6.

What is claimed is:

1. A composition for immunologically determining a HCV antibody in a specimen, which comprises an antigen or a peptide having substantially equivalent action thereto, wherein a protein antigen encoded by NS3 region on HCV genome or a peptide having substantially equivalent action thereto is used as an antigen, and said antigen is retained or converted so that it can substantially maintain a form of a reduced NS3 related antigen.

2. The composition of claim 1, wherein a cysteine residue is retained or converted so that it can react immunologically and specifically with said reduced HCV antibody, by either treatment of:

(1) Treating the reduced NS3 related antigen with a reducing agent to convert a cysteine residue existing therein into its reduced form, and maintaining the reduced NS3-related antigen in a dried state, in an inert gas atmosphere or in the presence of a deoxygenating agent;

(2) Modifying a thiol group of a cysteine residue existing in the reduced NS3-related antigen with a protecting or modifying agent for thiol group such as iodoacetamide, iodoacetic acid, p-chloromercuribenzoic acid, methyl iodide or a metal such as mercury, iron, lead and the like;

(3) Modifying a cysteine residue existing in the reduced NS3-related antigen by genetic recombination techniques such as site-directed mutagenesis to prepare a variant recombinant NS3 antigen;

(4) Reserving the reduced NS3-related antigen in the presence of an antioxidant until just prior to use;

(5) Treating the NS3 related antigen by cleaving a disulfide bond (-S-S-) existing in NS3-related antigen, and treating it with an enzyme such as disulfide reductase and the like that can form the thiol group; or

(6) Treating the NS3 related antigen with a reagent having an affinity for a cysteine residue present in the NS3-related antigen.

3. The composition of claim 1 or 2 wherein the antigen has been prepared, isolated and/or purified in a reducing condition or in the absence of any enzyme.

4. The composition of any one of claims 1-3 wherein the protein antigen encoded by NS3 region is 33C.

5. A method to immunologically determine a HCV antibody in a specimen using an antibody or a peptide having substantially equivalent action thereto, characterized in that a protein antigen encoded by NS3 region on HCV genome or a peptide having substantially equivalent action thereto is used as an antigen and that a cystein residue present in said antigen is retained or converted so that it can react immunologically and specifically with an anti-reduced HCV antibody.

6. The method of claim 5, characterized in that the cysteine residue present in said antigen is retained or converted so that it can react immunologically and

specifically with an anti-reduced HCV antibody, by either treatment of:

(1) Treating the reduced NS3 related antigen with a reducing agent to convert a cysteine residue existing therein into its reduced form, and maintaining the reduced NS3-related antigen in a dried state, in an inert gas atmosphere or in the presence of a deoxygenating agent;

(2) Modifying a thiol group of a cysteine residue existing in the reduced NS3-related antigen with a protecting or modifying agent for thiol group such as iodoacetamide, iodoacetic acid, p-chloromercuribenzoic acid, methyl iodide or a metal such as mercury, iron, lead and the like;

(3) Modifying a cysteine residue existing in the reduced NS3-related antigen by genetic recombination techniques such as site-directed mutagenesis to prepare a variant recombinant NS3 antigen;

(4) Reserving the reduced NS3-related antigen in the presence of an antioxidant until just prior to use;

(5) Treating the NS3 related antigen by cleaving a disulfide bond (-S-S-) existing in NS3-related antigen, and treating it with an enzyme such as disulfide reductase and the like that can form the thiol group; or

(6) Treating the NS3 related antigen with a reagent having an affinity for a cysteine residue present in the NS3-related antigen.

7. The method of claim 5 or 6 wherein the antigen has been prepared, isolated and/or purified in a reducing condition or in the absence of any enzyme.

8. The method of any one of claims 5-7 wherein the protein antigen encoded by NS3 region is 33C.

9. The method of claim 5 wherein said antigen reagent is reserved or used for the determination in a dried state or under a deoxygenating agent or an inert gas.

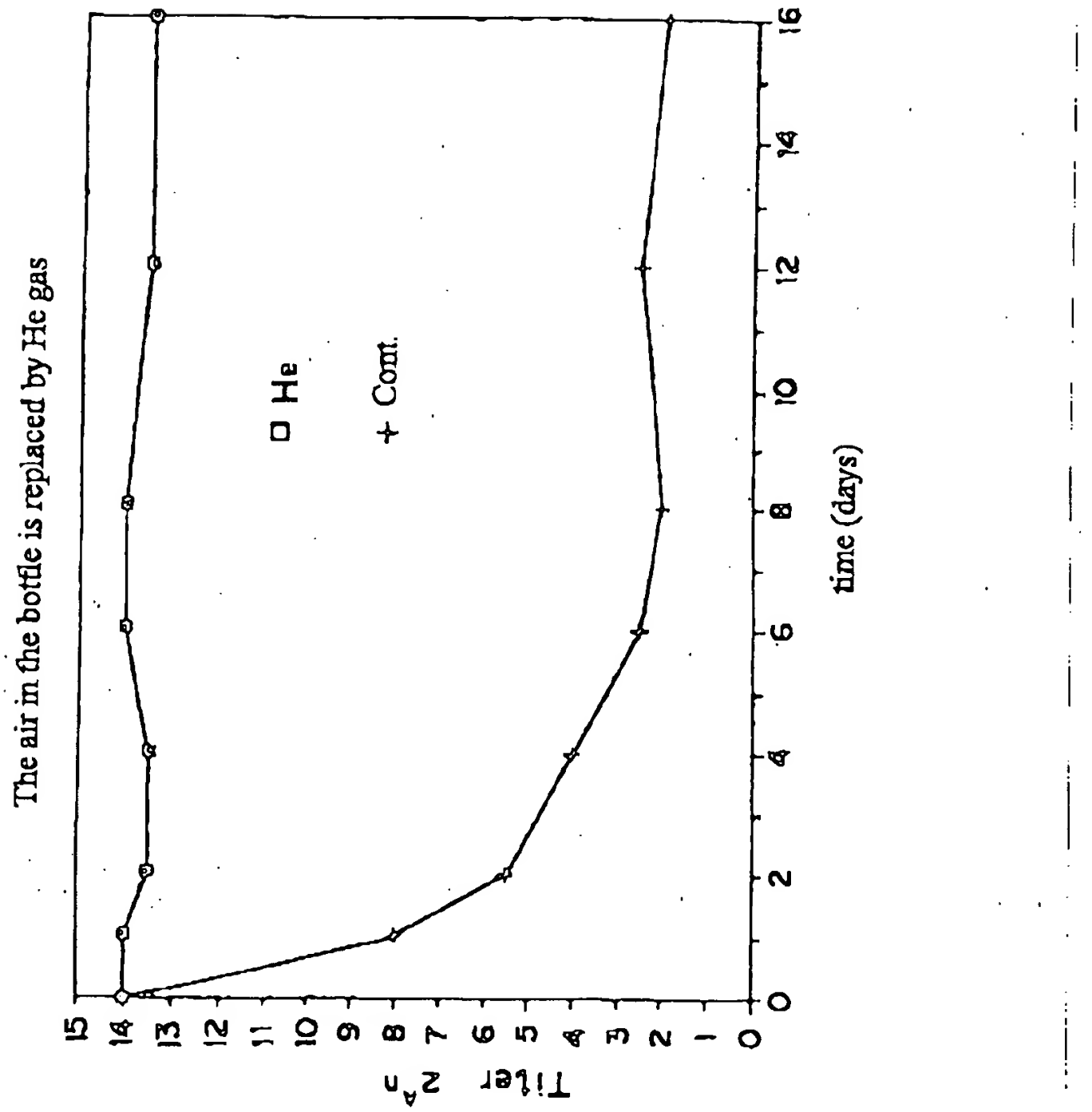

The air in the bottle is replaced by He gas
□ He
+ Cont.
Titer 2^{An}
time (days)
0 1 2 3 4 5 6 7 8 9 10 11 12 13 14 15
0 2 4 6 8 10 12 14 16

Fig. 1

Fig. 2 (a)

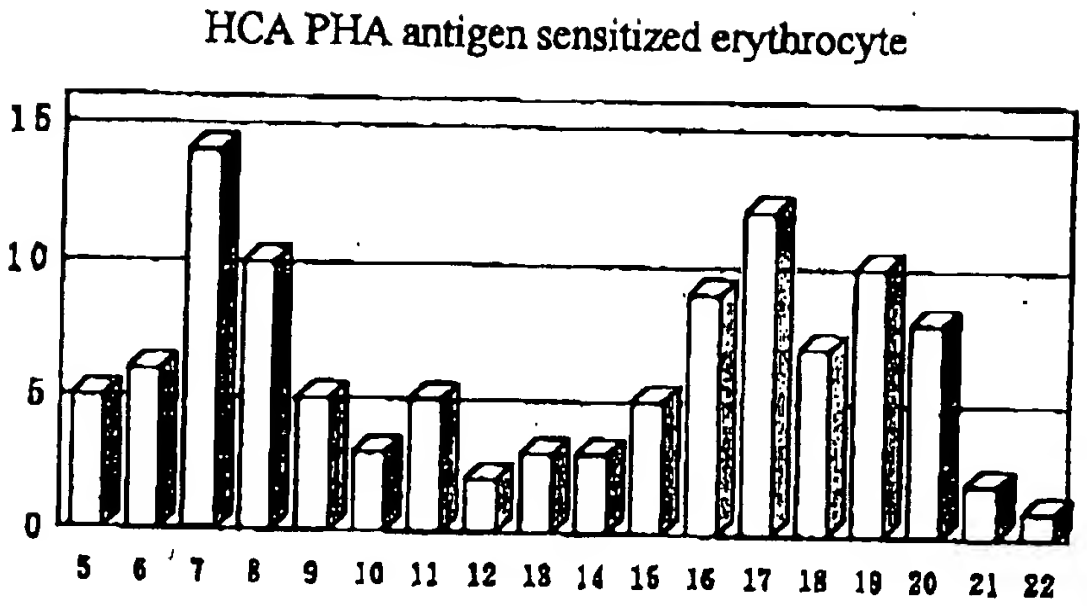

HCA PHA antigen sensitized erythrocyte
15
10
5
0
5 6 7 8 9 10 11 12 13 14 15 16 17 18 19 20 21 22
Titer (2^n)

Fig. 2 (b)

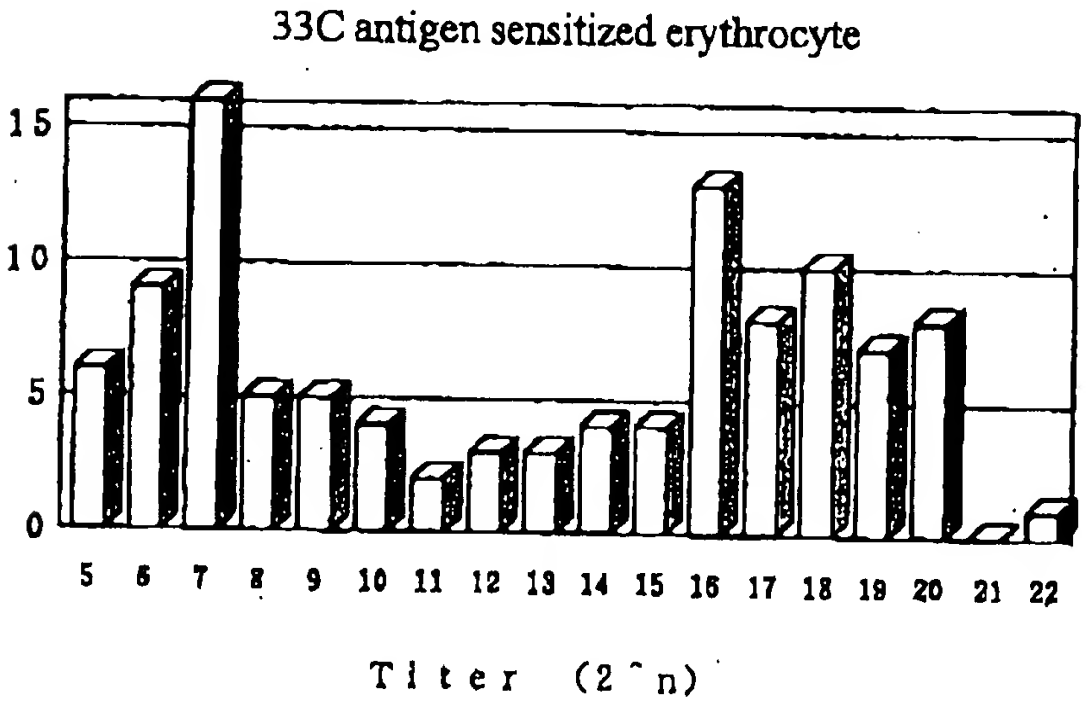

33C antigen sensitized erythrocyte
15
10
5
0
5 6 7 8 9 10 11 12 13 14 15 16 17 18 19 20 21 22
Titer (2^n)

Fig. 3 (c)

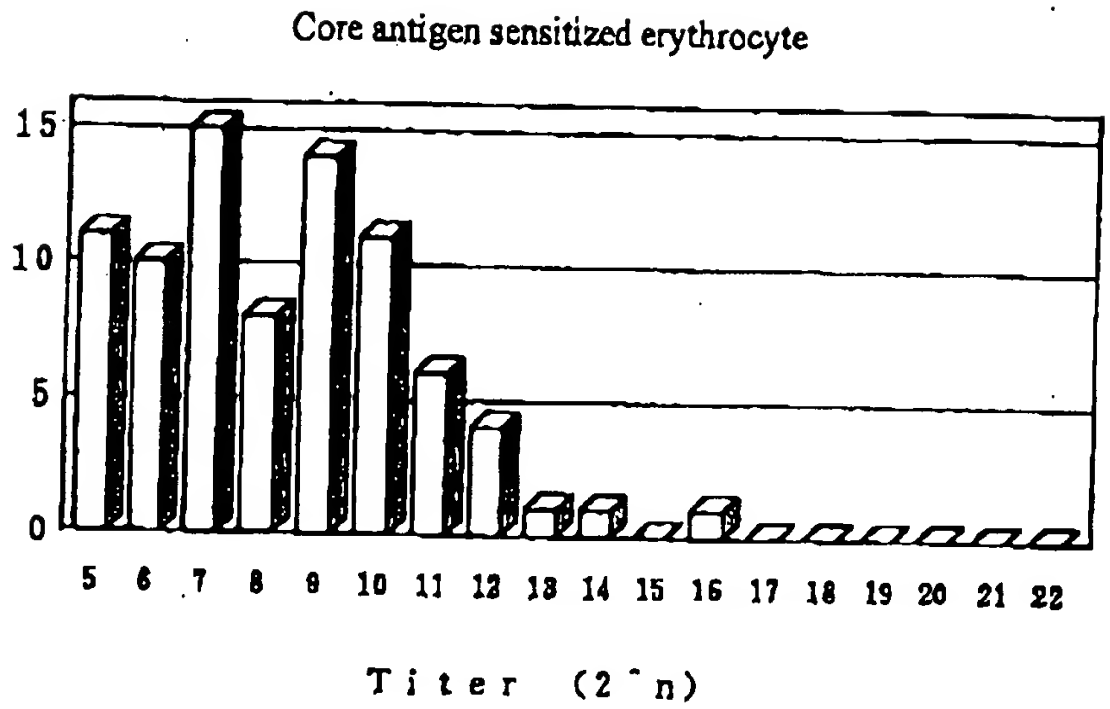

Core antigen sensitized erythrocyte
15
10
5
0
5 6 7 8 9 10 11 12 13 14 15 16 17 18 19 20 21 22
Titer (2^n)

Fig. 3 (d)

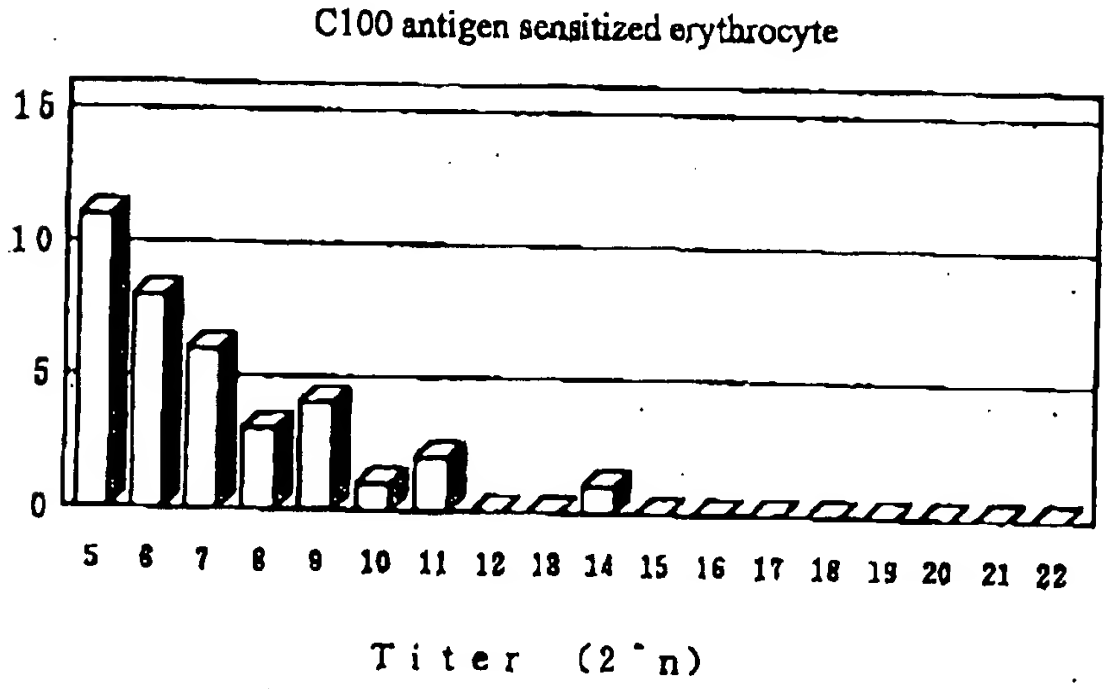
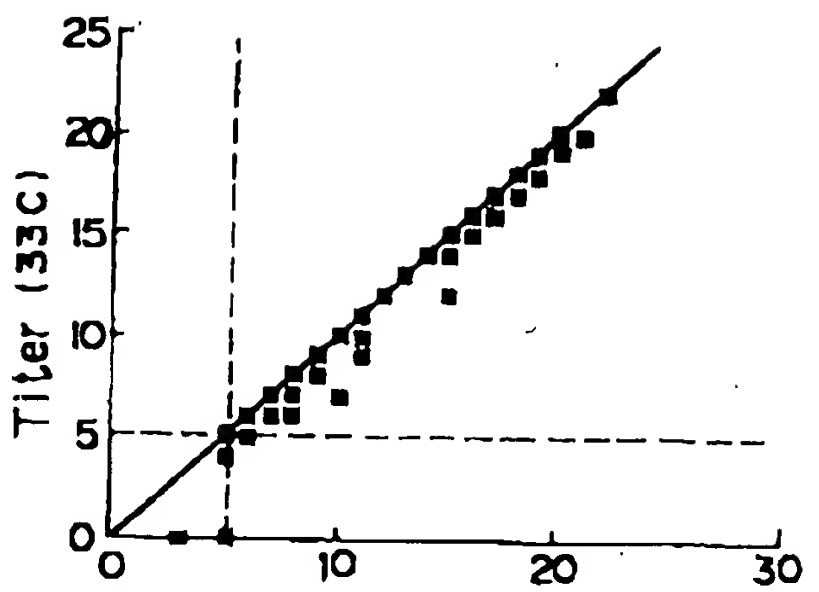

C100 antigen sensitized erythrocyte
15
10
5
0
5 6 7 8 9 10 11 12 13 14 15 16 17 18 19 20 21 22
Titer (2^n)

Fig. 4

(a)


25
20
15
10
5
0
Titer (33C)
0
10
20
30
Titer (33C, C100 and core mixed antigen)

(b)

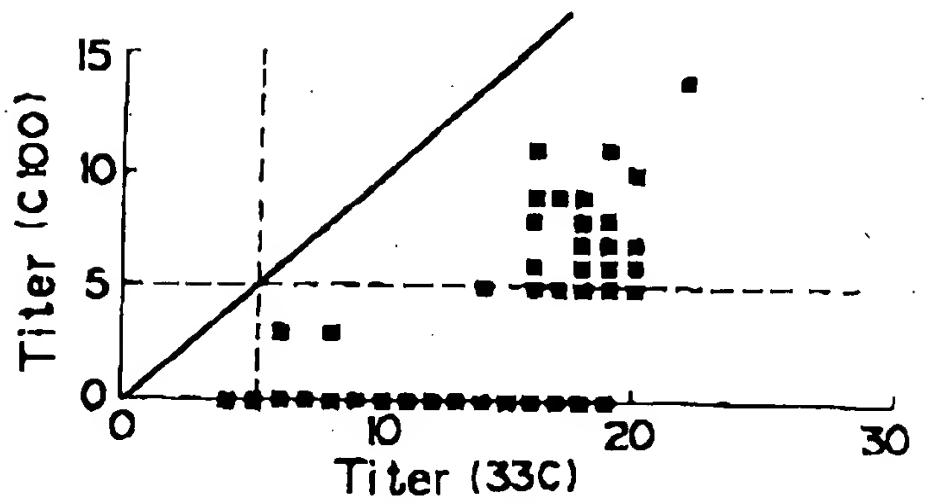

15
10
5
0
Titer (C100)
0
10
20
30
Titer (33C)

Fig. 5

(C)